Evidence That Stratum Corneum Chymotryptic Enzyme Is Transported to the Stratum Corneum Extracellular Space *Via* Lamellar Bodies

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Stratum corneum chymotryptic enzyme (SCCE) is a recently discovered human serine proteinase that may be specific for keratinizing squamous epithelia. SCCE has properties compatible with a function in the degradation of intercellular cohesive structures during stratum corneum turnover and desquamation. SCCE is expressed in suprabasal keratinocytes. In this study, we demonstrate the subcellular localization of SCCE in the upper granular layer, in the stratum corneum of normal non-palmoplantar skin, and in cohesive parts of hypertrophic plantar stratum corneum, using immunoelectron microscopy of ultrathin cryosections labeled with SCCE-specific monoclonal antibodies detected with gold-labeled secondary antibodies. A narrow zone close to the transition between the granular and cornified layers showed positive SCCE staining after fixation. By means of immunoelectron microscopy, SCCE was found in association with structures resembling intracellular lamellar bodies in the uppermost granular cells and in similar structures undergoing extrusion to the extracellular space between the uppermost granular cells and the lowermost cornified cells. In the stratum corneum, the detected SCCE was confined to the extracellular space and was found in association with intact and partially degraded desmosomes, as well as in the parts of the extracellular space devoid of desmosomes. We conclude that SCCE may be stored in lamellar bodies in the stratum granulosum and transported *via* these structures to the stratum corneum extracellular space. The results further support the idea that the physiologic function of SCCE may be to catalyze the degradation of desmosomes in the stratum corneum during remodeling of the deeper layers of this tissue, and at a later stage serve as a prerequisite for desquamation. ***Key words: epidermis/serine protease/desmosomes/desquamation.***
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Recent work has established that the modified desmosomes of the stratum corneum may have major importance for stratum corneum cell cohesion [1–4]. This implies that a well-regulated proteolytic degradation of intercellular cohesive structures is likely to be involved in normal stratum corneum turnover, eventually leading to a desquamation that balances *de novo* production of corneocytes. The nature of these mechanisms has been largely unknown.

Stratum corneum chymotryptic enzyme (SCCE) is a recently discovered serine proteinase. SCCE, which is generally present in human stratum corneum [5,6], has an inhibitor profile that suggests that it is the major proteinase involved in the desquamation-like unipolar cell shedding that can be observed in plantar stratum corneum *in vitro* [7,8]. There is indirect evidence that SCCE may be located extracellularly in the stratum corneum [9]. Although it is optimally active at pH 7–8, it is active also at pH 5.5 [5,6]. These findings have been taken as evidence that SCCE also may function in desquamation *in vivo*.

The recent purification of SCCE [10] has made it possible to raise SCCE-specific monoclonal antibodies (MoAbs). Immunohistochemical studies at the light microscopic level have shown that SCCE is expressed in upper suprabasal keratinocytes in the epidermis. In the hair follicle, it was found only in the inner root sheath. In the human oral cavity, SCCE could be detected only at sites where there is formation of a stratum corneum [11]. These studies thus gave evidence that SCCE may be specific for keratinizing squamous epithelia. However, it could not be concluded whether SCCE functions in keratinization or in desquamation [11].

We have studied the subcellular localization of SCCE using immunoelectron microscopy. We show that SCCE may be associated with the lamellar bodies in the keratinocytes of the stratum granulosum and transported *via* these structures to the stratum corneum extracellular space, where the enzyme is found in association with desmosomes. Thus, our findings further support a role for SCCE in the desquamation process.

MATERIALS AND METHODS

Rabbit anti-mouse immunoglobulins (Igs) and peroxidase-antiperoxidase reagents were purchased from Dakopatts (Hägersten, Sweden). Goat anti-mouse Igs conjugated with 10 nm colloidal gold particles were obtained from BioCell Research Laboratories (Cardiff, UK). The anti-human filaggrin mouse MoAb (MoAb filaggrin, IgG_1) was from PAESEL-LOREI (Frankfurt am Main, Germany; cat. no. 14-143-0056); the MoAb

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Abbreviations: PB, 0.1 M sodium phosphate, pH 7.4; PFA, paraformaldehyde; SCCE, stratum corneum chymotryptic enzyme.

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specific for human pregnancy zone protein (IgG_1 κ [12]) was a gift from Dr. T. Stigbrand (Umeå, Sweden). The SCCE-specific mouse MoAb TE4B and MoAb TE9B (both IgG_1 κ) are described elsewhere [11]. Diaminobenzidine was from Sigma (St. Louis, MO).

Tissues Three-millimeter punch biopsies (for light microscopy) or approximately 1 cm^2 shave biopsies were taken under local anesthesia from the upper gluteal region of volunteers (three men and one woman, ages 26–54 years) with normal skin. In addition, normal skin was examined from two women (ages 22 and 50 years) undergoing mammary reduction surgery. Hypertrophic, normal plantar stratum corneum was taken as thin slices with a scalpel from the inferiolateral aspect of the heels of two healthy male volunteers (ages 29 and 47 years).

Preparation of Tissues for Light Microscopy Skin samples were either immediately mounted in Tissue-Tek O.C.T. compound (Miles Laboratories Inc., Elkhart, IN) and snap frozen in propane chilled with liquid nitrogen, or first fixed for 1 h at room temperature in 2% paraformaldehyde (PFA) in 0.1 M sodium phosphate, pH 7.4 (PB) before embedding and snap freezing.

Preparation of Tissues for Electron Microscopy The skin samples were immediately immersed in 2% PFA in PB and minced to pieces not exceeding 1 mm^3 in size. The fixation was allowed to proceed for 1 h at room temperature, followed by immersion of the tissue pieces in 2.3 M sucrose in PB for 30 min at room temperature. Single specimens were then transferred to brass pins, snap frozen in liquid nitrogen, and stored in liquid nitrogen until further processed.

Immunostaining for Light Microscopy Freeze-cut, 7-μm sections were fixed in acetone and immunostained with the peroxidase-antiperoxidase method [13] with MoAb TE4B or MoAb TE9B, 10 μg/ml, as described previously [11]. Sections of samples fixed in PFA were incubated with 0.15% glycine in PB for 15 min before staining. Dilution of antibodies and reagents and rinses were performed according to one of two protocols: 1) Except for one 5-min rinse after each of the incubations with primary and secondary antibodies, which was carried out with PB, all dilutions and rinses were done with 0.1 M Tris-HCl, pH 7; 2) all dilutions and rinses were carried out with PB.

Cryoultramicrotomy and Immunolabeling for Electron Microscopy Ultrathin (70–100 nm) cryosections were transferred to formvar-carbon–coated gold grids. Immunolabeling with primary antibodies diluted in PB with 0.15% glycine, 0.5% bovine serum albumin, and 5% normal goat serum was carried out at room temperature for 1 h. Bound antibodies were detected with gold-labeled goat anti-mouse Igs diluted 1/20 in PB with 0.15% glycine and 0.5% bovine serum albumin for 30 min at room temperature. After post-fixation in 2.5% glutaraldehyde in PB, the sections were counterstained with uranyl oxalate and uranyl acetate, embedded in methyl cellulose [14], and air dried. Some sections were counterstained with 2% ammonium molybdate in double-distilled water for 20 seconds, followed by air drying.

MoAb TE4B and MoAb TE9B were used at a concentration of 10 μg/ml. MoAb filaggrin (positive control) was used in dilution 1/100, and the MoAb for pregnancy zone protein (negative control) at 12 μg/ml. Blank incubations with antibody dilution buffer were also used as negative controls. Stained sections were examined in a JEOL 1200 EX II electron microscope at 60–100 kV.

RESULTS

Evaluation of the Effect of Fixation on Staining with SCCE-Specific Antibodies The effects of PFA fixation and buffer composition on the staining of normal epidermis with the available SCCE-specific MoAbs were studied in an experiment presented in **Fig 1**. MoAb TE4B and MoAb TE9B gave identical results. With a staining procedure previously found [11] to be optimal for sections from unfixed tissues, the antibodies gave the most intense staining of the cells in the stratum granulosum, but there was staining also in the upper part of the spinous layer **(Fig 1*A*)**. **Figure 1*B*** shows that when PB was used throughout the staining procedure with unfixed tissue, there was no staining of the epidermis. In the tissue fixed with PFA, there was no specific staining of the basal cells and most of the spinous layer, but there was still staining of cells in the granular layer. There was also weak, irregular staining of the stratum corneum **(Fig 1*C,D*)**. In contrast to unfixed tissue, use of PB throughout the staining procedure did not seem to affect the SCCE-specific staining of PFA-fixed tissue **(Fig 1*D*)**.

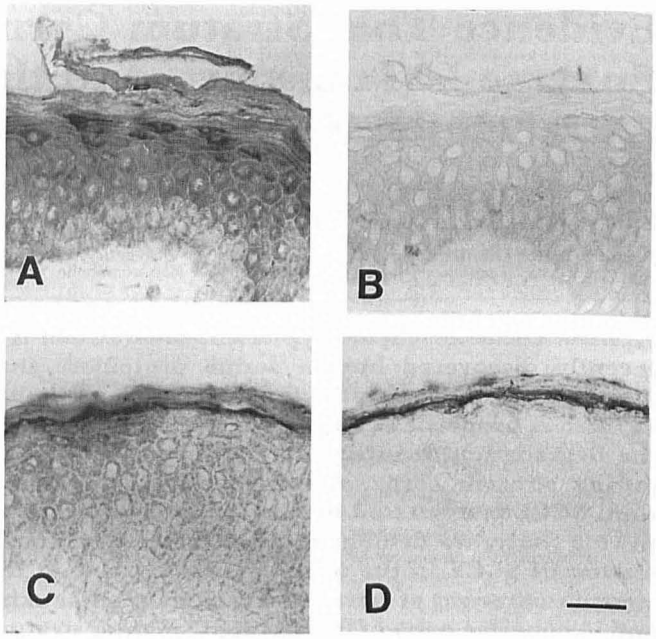

Figure 1. The effect of ionic strength and fixation on SCCE staining. Light microscopy of normal human non-palmoplantar epidermis labeled with MoAb TE4B. *A,B*, unfixed tissue; *C,D*, tissue fixed with PFA. *A,C*, staining according to protocol 1; *B,D*, staining according to protocol 2 (see *Materials and Methods* for details). *Bar*, 25 μm.

Subcellular Localization of SCCE The subcellular localization of SCCE in the transition zone between the granular and cornified layers in non-palmoplantar epidermis is shown in **Fig 2*A***. **Figure 2*B*** shows a negative control, stained with the MoAb for pregnancy zone protein, and **Fig 2*C*** shows a positive control stained with MoAb filaggrin. With the SCCE-specific antibody, gold particles were found in structures that appeared to bulge into or to cause a widening of the extracellular space between the lowermost corneocyte and the uppermost granular cell. In the stratum corneum, SCCE appeared to be confined to the extracellular space. The label was found between desmosomes, in association with desmosomes apparently undergoing degradation, and sometimes in association with apparently intact desmosomes. At higher magnification, the immunolabeled widened part of the extracellular space between the uppermost granular cells and the lowermost cornified cells had a multivesicular appearance **(Fig 3)**. In the stratum granulosum **(Fig 4)**, SCCE-specific gold labeling was seen predominantly in association with membrane-surrounded structures. These were irregular in shape and appeared to consist of aggregates of smaller, 30–100-nm rounded structures. In the uppermost granular cells, the label was found most often apically **(Fig 4*A*)**. One cell layer down in the stratum granulosum, labeled membrane-surrounded structures were sometimes seen close to the apical cell border **(Fig 4*B*)** but were also found at some distance from the upper cell margin **(Fig 4*C*)**.

Figure 5*A* shows the distribution of SCCE in non-palmoplantar stratum corneum at a level approximately halfway between the stratum granulosum and the skin surface. In this part of the tissue, SCCE was found mainly in association with still apparently intact desmosomes, but sometimes also in the desmosome-free intercellular space. In cohesive parts of the plantar stratum corneum **(Fig 5*B*)**, the major part of the detectable SCCE was confined to the extracellular space between desmosomes.

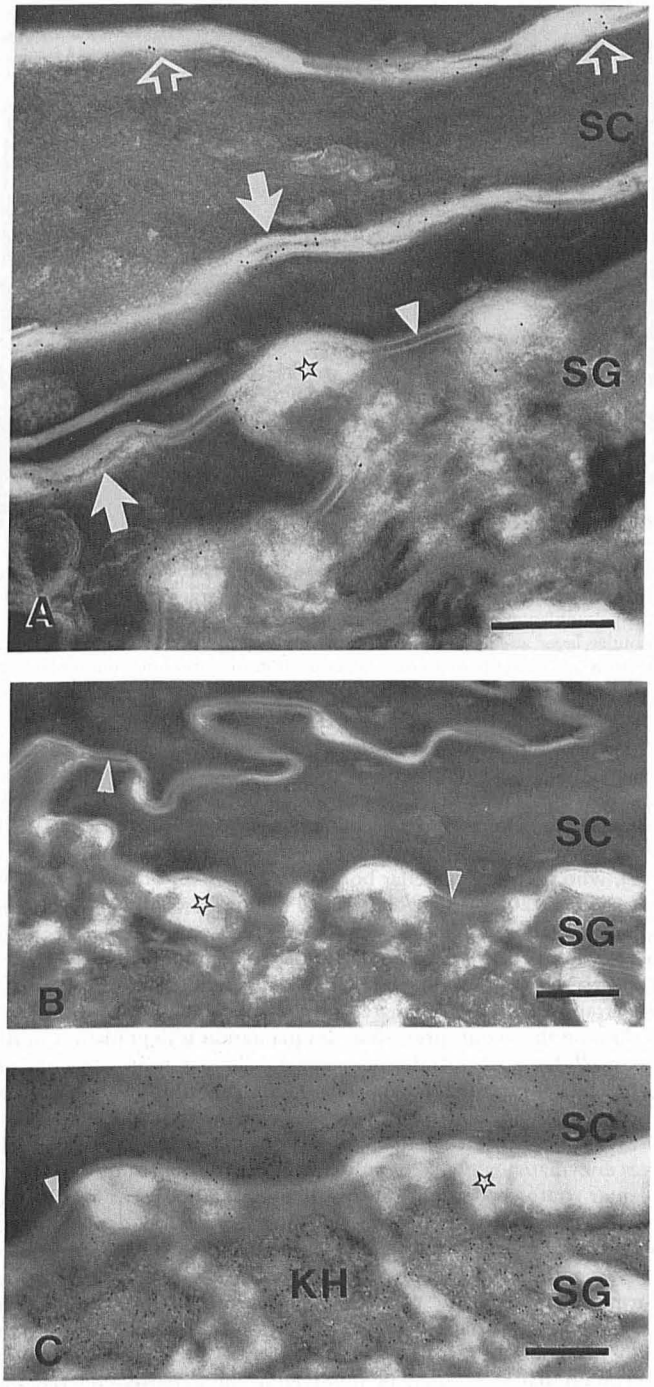

Figure 2. Immunogold labeling of SCCE and controls. Electron micrographs of immunolabeled normal human non-palmoplantar epidermis, counterstained with uranyl oxalate/uranyl acetate. The first antibody was MoAb TE4B (*A*), the MoAb for pregnancy zone protein (*B*), and MoAb filaggrin (*C*). SC, stratum corneum; SG, stratum granulosum; KH, keratohyalin; *white arrowheads*, apparently intact desmosomes; *white arrows*, partially degraded desmosomes; *open arrows*, intercellular space with no desmosomes; *star*, widening of the intercellular space between granular and cornified layers. *Bars*, 500 nm.

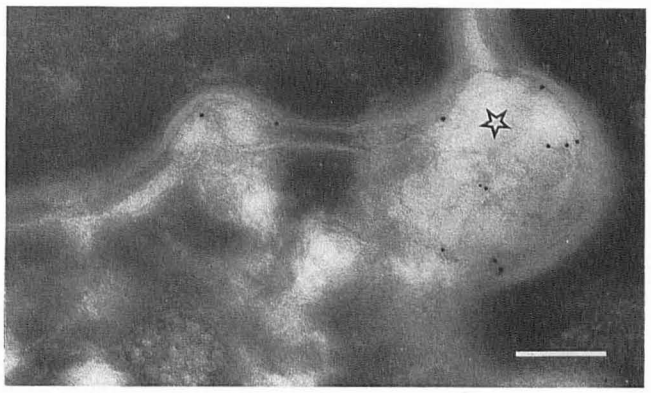

Figure 3. SCCE in the extracellular space between the stratum granulosum and the stratum corneum. Electron micrograph of normal human non-palmoplantar epidermis immunolabeled with MoAb TE4B and counterstained with uranyl oxalate/uranyl acetate. *Star*, widening of the intercellular space between granular and cornified layers. *Bar*, 200 nm.

DISCUSSION

In the present work, we have shown that SCCE-specific monoclonal antibodies bind to membrane-surrounded structures, which are apparently located within the cells of the stratum granulosum. These membrane-surrounded structures were also seen in those parts of the transition zone between the stratum granulosum and the stratum corneum, where lamellar bodies are known to be extruded to the extracellular space [15,16]. The ultracryotechnique and the mild fixation necessary to preserve SCCE-specific epitopes precluded a definite identification of lamellar bodies in our micrographs. Furthermore, we can not entirely rule out the possibility that the membrane-surrounded structures in the stratum granulosum may be invaginations of the plasma membrane surrounding secreted lamellar bodies. However, this seems unlikely because we could not find vesicular structures, such as those shown in **Figs 3 and 4**, or SCCE-specific gold labeling in the extracellular space between two granular cells. Even though we cannot definitely state that SCCE is stored in intracellular lamellar bodies in the stratum granulosum, our results strongly suggest that this is the case and that SCCE is secreted *via* lamellar bodies to the extracellular space in the later stages of keratinization. In the stratum corneum, SCCE was found exclusively in the extracellular space, in association with desmosomes as well as in areas with no desmosomes or with desmosomal remnants.

The immunoelectron microscopic experiments may not, however, give a complete picture of the subcellular localization of SCCE in the epidermis. We have previously found [11] that SCCE-specific staining of acetone-fixed epidermis is dependent on the ionic strength of the buffer used. When 0.1 M Tris-HCl, pH 7, was used for all antibody dilutions and rinses except for one 5-min rinse in a buffer with physiologic ionic strength, specific staining was obtained. When a buffer with physiologic ionic strength was used throughout the staining procedure, no staining was obtained. The fact that staining was abolished after pretreatment of the sections at higher ionic strength, before incubation with primary antibody diluted in 0.1 M Tris-HCl pH 7, was taken as evidence that SCCE was solubilized at ionic strengths exceeding that of the 0.1-M Tris buffer [11]. As shown in the light microscopic studies in this work, fixation with PFA resulted in an increased resistance of the antibody-binding molecules to extraction with buffers of moderate ionic strength. However, it also caused a decrease in staining intensity with the peroxidase-antiperoxidase method as compared with unfixed tissue. One explanation is that the areas that were stained after PFA fixation were those containing the highest amounts of SCCE. Another explanation, however, may be that SCCE exists in several forms or in several compartments with different sensitivities to PFA fixation. Hypothetically, SCCE in the lipid-rich surroundings of the lamellar bodies and in the stratum corneum extracellular space could be more resistant to fixation than SCCE at other locations.

To our knowledge, SCCE is the first hydrolytic enzyme demonstrated by means of immunoelectron microscopy to be associated with lamellar bodies and to be present in the stratum corneum

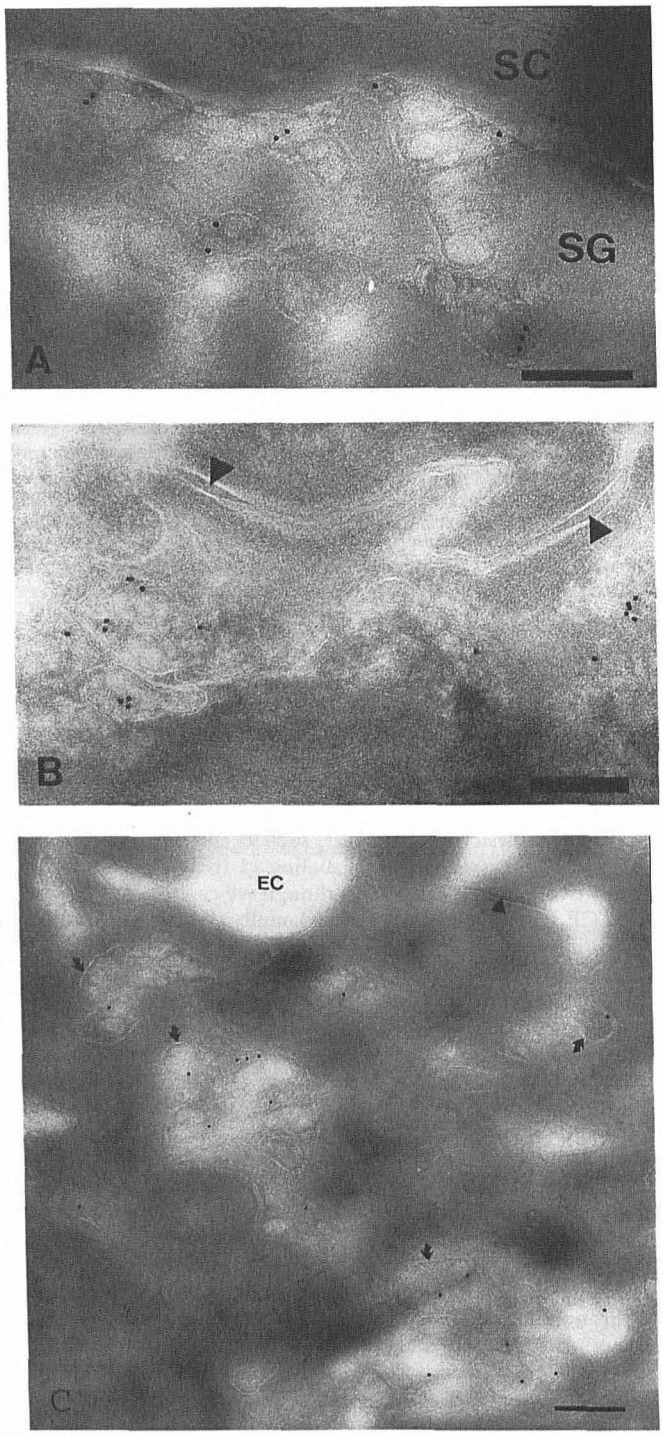

Figure 4. SCCE in the upper stratum granulosum. Electron micrographs of normal human non-palmoplantar epidermis immunolabeled with MoAb TE4B and counterstained with ammonium molybdate (*A*,*C*) and uranyl oxalate/uranyl acetate (*B*). *A*, apical area of uppermost granular cell; *B*, apical area of granular cell one cell layer down from the stratum corneum; C, granular cell one cell layer down from the stratum corneum. SC, stratum corneum; SG, stratum granulosum; EC, extracellular space; *arrowheads*, desmosomes; *arrows*, membrane-surrounded structures. *Bars*, 200 nm.

extracellular space in close association with desmosomes. Our findings corroborate those of several other groups, supporting the idea that lamellar bodies may serve as a delivery vehicle for hydrolytic enzymes to the stratum corneum extracellular space.

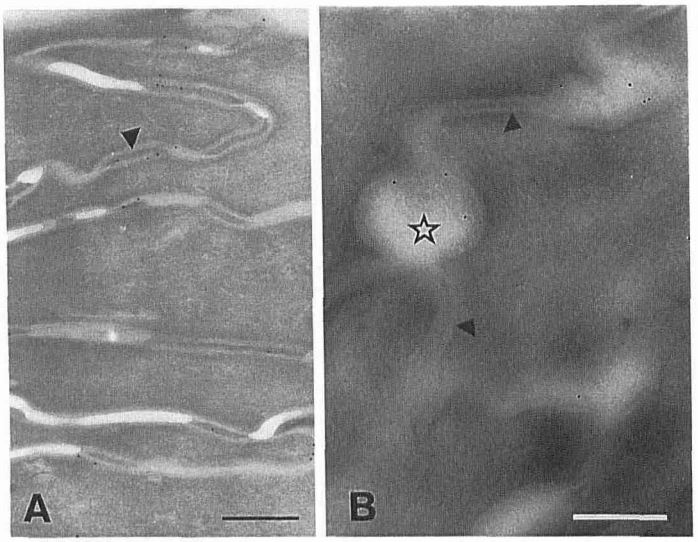

Figure 5. SCCE in upper non-palmoplantar stratum corneum and plantar stratum corneum. *A*, electron micrograph of normal human non-palmoplantar stratum corneum approximately halfway between the granular layer and the skin surface immunolabeled with MoAb TE4B. *B*, electron micrograph of human plantar stratum corneum immunolabeled with MoAb TE4B. Counterstaining was with uranyl oxalate/uranyl acetate. *Arrowheads*, desmosomes; *star*, intercellular space between desmosomes. *Bars*, 400 nm.

Freinkel and Traczyk [17,18] found an enrichment of acid hydrolases in subcellular fractions of fetal rat epidermis enriched with lamellar granules. Similar results were reported by Grayson *et al* [19], who prepared lamellar bodies from the skin of neonatal mice. Menon *et al* [20], using cytochemical techniques, showed lipase and sphingomyelinase activities in the stratum corneum intercellular space. In electron microscopic studies, Allen and Potten [21] and Fartasch *et al* [16] found evidence of degradation of desmosomes in the stratum corneum mediated by the lamellar body content.

Among the events preceding desquamation is degradation of the extracellular parts of desmosomes in the stratum corneum. A proteinase responsible for this degradation should thus be localized preferentially in the extracellular space of the stratum corneum. In previous studies on plantar corneocytes dissociated *in vitro*, there was enzymatic evidence of an extracellular localization of SCCE in plantar stratum corneum [9]. In the present work, this localization of SCCE in the stratum corneum has been directly demonstrated. Furthermore, our studies give evidence of a close association between SCCE and the desmosomes of the stratum corneum. This further supports a role of SCCE in stratum corneum turnover and desquamation. As discussed elsewhere [11], this may not be the only function of SCCE. For instance, at present it cannot be excluded that SCCE may be involved in the extensive degradation of cellular structures that takes place during the terminal stages of epidermal differentiation. For a further understanding of the functions of SCCE, several questions must be answered. For instance, because SCCE may be produced by the keratinocytes as an inactive precursor [22], it will be crucial to find out where in the tissue the transformation to active SCCE takes place.

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